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AN EMBRYO STAGING SYSTEM FOR COMPARISON OF ZYGOTIC AND SOMATIC EMBRYO DEVELOPMENT

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Abstract

An embryo classification system has been developed based on observations of Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) and Pinus taeda L. (loblolly pine) zygotic embryos and on the literature concerning embryology within the pine family. This system is suitable for classification of both zygotic and somatic embryos of loblolly pine, Picea abies L. (Norway spruce), and Douglas-fir. This system can be used to compare early, mid, and late stages of zygotic and somatic embryos. The use of this system has been found to help in the formation of embryo development protocols for loblolly pine. In addition, this system has been useful in staging zygotic embryos for physiological and biochemical studies. Loblolly pine somatic embryos from liquid medium cell suspensions and from subsequent development on semisolid medium are compared to zygotic embryos.

Introduction

The U.S. forest products industry accounts for nearly 7% of the total U.S. manufacturing output. However, much of the industry faces reduced availability of future timber resources. If the industry is to continue to grow, it must secure reliable sources of raw material. Clonal propagation of high-value, fast-growing trees in a crop-like setting offers the potential to meet future needs for high quality raw materials.

Somatic embryogenesis is a type of plant tissue culture which starts with a piece of donor plant and forms new embryos. In conifers, somatic embryogenesis currently

involves the culture of zygotic seed embryos, usually from breeding programs, to start or initiate a culture. The major advantage of this technology lies in its ability to rapidly multiply highly valuable genetic material forming an unlimited number of identical somatic seedlings. However, a major limiting factor for the scientific or commercial use of somatic embryogenesis is found in the quality of embryos produced. Few, if any, somatic embryogenesis systems in coniferous plant species or angiosperms produce embryos similar in biochemistry or vigor to zygotic embryos. Many embryogenic systems appear to produce somatic embryos which are capable of germination, but which do not fully mature, resulting in slow germination and initial growth.

In 1990 researchers at the Institute of Paper Science and Technology began an approach toward embryo quality improvement which focused on the comparison of zygotic and somatic embryo development. It was expected that somatic embryo quality would improve through imitation of the hormonal, nutritional, and physical environments during zygotic embryo development. The first step in this approach was to develop a zygotic embryo classification system which was based on the embryology literature within the pine family yet was detailed enough to meet the need of closely following zygotic and somatic embryo growth.

Descriptions of the developmental stages which occur during conifer zygotic and somatic embryogenesis have been reviewed (1,2,3,4,5). However, stage descriptions were not usually classified according to the literature on conifer embryo development (5) and were not detailed enough to meet the needs of somatic embryo characterization for comparison to zygotic embryos.

Since overall embryo development is similar within the pinaceae (6), one comprehensive scheme should work for most or all species within the family. In addition, this scheme could be used to categorize and uniformly classify somatic embryos. The goal of this study was to classify the major developmental stages of Douglas-fir and loblolly pine zygotic embryos in order to develop an embryo

staging model that could be applied to zygotic and somatic embryos within the pine family. At the same time, the model needed to be in accord with classical work on conifer embryology (6,7). We present here a novel classification system which recognizes additional categories of embryo staging enabling a more comprehensive evaluation of embryo development.

Materials and Methods

Cones of Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) were collected throughout the summer of 1990. Cones of Pinus taeda L. (loblolly pine) were collected throughout the summer of 1993 and from Brazil during January 1994. Embryos were dissected from the female gametophyte and classified using a modification of the Buchholz and Stiemert, 1945 (7) system. These authors did not classify the proembryo and the first stage of early embryo development. Consequently, we have arranged nine stages while Buchholz and Stiemert, 1945 (7) had seven. In addition, we checked to be sure that our classifications were in agreement with Allen and Owens, 1972 (6).

Cultures of somatic embryos from loblolly pine were initiated as described by Becwar et al. 1990 (8), or with modifications in media mineral composition. Somatic embryos were grown in cell suspension culture medium 16 (Table 1) on a rotary shaker at approximately 90 RPM. Cultures were transferred on a weekly subculture cycle with a ratio of one part of settled cells plus nine parts of liquid medium. A sample of cultures was characterized for early-stage embryos present and plated onto semisolid development medium (24, Table 1) in order to evaluate a culture's potential to develop cotyledonary embryos. Several of the cultures producing cotyledonary embryos were later plated on an improved maturation medium, medium 240 (Table 1). As embryos developed, observations were made of the stages present.

Results

Zygotic Embryos

Stage descriptions are described below. References to literature and specific figures in the literature are contained in parentheses.

Stage 1. Proembryos from the free nucleate stage to embryos of approximately 12 cells with the earliest stage of suspensor elongation. The embryo proper is indistinct and translucent. The suspensor is not enlarged radially and consists of the original number of cell files. The embryo is composed of 3-4 horizontal "tiers" (Allen and Owens, 1972, Figures 6.1 A-G and 6.2 G-H).

Stage 2. The embryo proper contains more than 12 cells and the suspensor has elongated but has not increased radially. The embryo is still at the micropylar end of the megagametophyte. The embryo proper is still microscopic but is distinct and translucent (Allen and Owens, 1972, Figures 6.1 H-J, 6.2 I-J, and 6.3 A-D).

Stage 3. The suspensor has increased radially and elongated such that the embryo proper is often found at the chalazal end of the corrosion cavity. The embryo proper is beginning to become white and opaque (Allen and Owens 1972, Figures 6.1 J-K and 6.3 E; Buchholz and Stiemert, 1945, stage I).

Stage 4. The suspensor has greatly expanded and both the embryo and suspensor have enlarged radially. The embryo proper has also enlarged longitudinally and has become opaque. The apex of the embryo proper is dome-shaped (Allen and Owens, 1972, Figures 6.1 K-M and 6.3 E; Buchholz and Stiemert, 1945, stage II.)

Stage 5. Similar to stage 4, but the apical meristem primordium is clearly visible (Allen and Owens, 1972, Figure 6.3 F; Buchholz and Stiemert, 1945, stage III).

Stage 6. Similar to stage 5, but cotyledon primordia are barely visible below the shoot apical meristem (Allen and Owens, 1972, Figures 6.1 N and 6.3 F; Buchholz and Stiemert, 1945, stage IV).

Stage 7. Similar to stage 6 with cotyledons that have elongated but do not overtop the

shoot apical meristem as seen in transverse view (Allen and Owens, 1972, Figures 6.1 N and 6.3 F; Buchholz and Stiemert, 1945, stage V).

Stage 8. Similar to stage 7, but the elongated cotyledons overtop the shoot apical meristem. Cotyledons have not closed, and the shoot apical meristem is still visible when viewed at a right angle from above (Buchholz and Stiemert, 1945, stage VI).

Stage 9. Similar to stage 8, but cotyledons have curved and have become joined at their tip so that the shoot apical meristem is completely enclosed and cannot be seen without excision of cotyledons (Allen and Owens, 1972, Figure 6.3 I; Buchholz and Stiemert, 1945, stages VII and VIII).

Somatic Embryos

Table 2 shows the characterization of embryo appearance for 25 loblolly pine genotypes grown in medium 16. Approximately 25% of the genotypes tested showed the presence of some embryo head organization into stages greater than one. When a sampling of genotypes with or without early-stage embryo organization were transferred to development and maturation medium 24, only those cultures with stages greater than one formed cotyledonary embryos. It therefore appears important to evaluate somatic embryos for stage in order to avoid moving cultures forward in the development protocol when they are unlikely to continue embryo growth. It may be important to observe cultures for embryo stage and move them to the next developmental medium only when embryo stage is maximal.

Stages 1, 2, and 3 were present in cell suspensions in medium 16. Somatic embryos at stages 4-8 were observed on semisolid medium 240. There was a high degree of similarity between zygotic and somatic embryo stages. However, as somatic embryo stages approach stage 4 or greater, somatic embryos were generally smaller than zygotic embryos. To date we have not observed stage 9 somatic loblolly pine somatic embryos.

Discussion

This classification system has proved useful for Douglas-fir, loblolly pine, and Norway spruce zygotic embryos and loblolly pine and Norway spruce somatic embryos. Embryo stages are easy to recognize; ratings are reproducible between evaluators; and the ratings are useful for identification of stage-specific developmental requirements. A similar system was also appropriate for white spruce (5). Any comparative classification system is based on ideal prototypes. However, some of the stages are hard to classify objectively. This is particularly true for stages 3 and 4 plus 8 and 9. One problem is that the size of the embryo is sometimes inconsistent with the morphological stage. Furthermore, not all of the above stages may occur in embryogenic tissues. To be really accurate in assessing developmental stages, quantitative measurements should be taken (8,7). However, this is a good rapid system for estimating the developmental stages of zygotic embryos. Furthermore, this scheme also works well with somatic embryos. Consequently, we view this embryo staging technique as a useful tool which can greatly assist in the development of reliable protocols for the production of high-quality somatic embryo which resemble zygotic embryos.

The observation that only early-stage embryos with morphological organization continue on to produce cotyledonary embryos suggests that a stage-related criterion must be met before embryo development will continue. This observation suggests that we focus research on obtaining organized structures in unresponsive cultures and avoid placement of unorganized cultures into the next development steps of the protocol. In addition, the rapid production of high-quality somatic embryos is essential for us to avail ourselves of the advantages which biotechnology offers the forest products industry. The propagation of elite genotypes and the exploitation of genetic engineering techniques require a tissue culture system capable of producing large volumes of desired materials. By identifying critical stage-related developmental needs, our classification system will better enable us to handle plant tissues more efficiently and to realize the potential

of these new technologies.

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Table 1. Composition of liquid culture maintenance medium (16), development and maturation medium (24), and improved development and maturation medium (240).

Components	Media (mg/L)		
	16	24	240
NH_4NO_3	603.8	–	200
KNO_3	909.9	100	909.9
$MgSO_4 \cdot 7H_2O$	246.5	370	246.5
KH_2PO_4	136.1	170	136.1
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	–	236.2
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	–	256.5
$MgCl_2 \cdot 6H_2O$	101.7	–	101.7
KCl	–	745	–
$CaCl_2 \cdot 2H_2O$	–	440	–
KI	4.15	0.83	4.15
H_3BO_3	15.5	6.2	15.5
$MnSO_4 \cdot H_2O$	10.5	16.9	10.5
$ZnSO_4 \cdot 7H_2O$	14.4	8.6	14.4
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.25	0.125
$CuSO_4 \cdot 5H_2O$	0.125	0.025	0.125
$CoCl_2 \cdot 6H_2O$	0.125	0.025	0.125
$FeSO_4 \cdot 7H_2O$	6.95	27.8	13.9
Na_2EDTA	9.33	37.3	18.65
Maltose	–	60,000	20,000
Sucrose	30,000	–	–
Polyethylene glycol MW 8,000	–	–	130,000
myo-Inositol	1,000	100	100
Casamino acids	500	–	500
L-Glutamine	450	1450	450
Thiamine•HCl	1.0	0.1	1.0
Pyridoxine•HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2.0	–	2.0
2,4-D	1.1	–	–
BAP	0.45	–	–
Kinetin	0.43	–	–
ABA	–	5.2	5.2
Gelrite	–	2,500	2,500
pH	5.7	5.8	5.7

Table 2. Characterization of liquid culture collection (25 genotypes) based on early-stage embryo appearance in medium 16.

No organization - single cells or clumps of cells (stage 1)

Genotypes: 2, 6, 81, 84, 114, 142, 175, 176

Eight genotypes (32%)

Mostly unorganized cell clumps at stage 1, occasional solar or polar embryo present at stages 1-2

Genotypes: 8, 18, 35, 48, 70, 80, 103, 111

Eight genotypes (32%)

Solar embryos present in abundance (clusters of embryos between stages 1-2)

Genotypes: 11, 23

Two genotypes (8%)

Polar embryos present in abundance

Seven genotypes (28%)

Stages 1-2

Genotype 118

Stage 2

Genotype 41, rough embryo heads
Genotype 37, smooth embryo heads
Genotype 38, smooth embryo heads
Genotype 71, smooth embryo heads

Stages 2-3

Genotype 31, small smooth heads, larger rough heads
Genotype 195, small and large smooth heads
